Positively charged retinoids are potent and selective inhibitors of the trans-cis isomerization in the retinoid (visual) cycle

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In vertebrate retinal photoreceptors, photoisomerization of opsin-bound visual chromophore 11-*cis*-retinal to *all-trans*-retinal triggers phototransduction events. Regeneration of the chromophore is a critical step in restoring photoreceptors to their dark-adapted state. This regeneration process, called the retinoid cycle, takes place in the photoreceptor outer segments and in the retinal pigmented epithelium (RPE). We have suggested that the regeneration of the chromophore might occur through a retinyl carbocation intermediate. Here, we provide evidence that isomerization is inhibited by positively charged retinoids, which could act as transition state analogs of the isomerization process. We demonstrate that retinylamine (Ret-NH_2) potently and selectively inhibits the isomerization step of the retinoid cycle *in vitro* and *in vivo*. Ret-NH_2 binds a protein(s) in the RPE microsomes, but it does not bind RPE65, a protein implicated in the isomerization reaction. Although Ret-NH_2 inhibits the regeneration of visual chromophore in rods and, in turn, severely attenuates rod responses, it has a much smaller effect on cone function in mice. Ret-NH_2 interacts only at micromolar concentrations with retinoic acid receptor, does not activate retinoid-X receptor, and is not a substrate for CYP26s, the retinoic acid-metabolizing cytochrome P450 enzymes. Ret-NH_2 can be a significant investigational tool to study the mechanism of regeneration of visual chromophore.

retinal | rhodopsin | vitamin A | electroretinogram | retinal pigment epithelium

In vertebrate photoreceptor cells, a photon causes isomerization of the 11-*cis*-retinylidene chromophore to *all-trans*-retinylidene, coupled to the visual opsin receptors. This photoisomerization triggers conformational changes of opsins that, in turn, initiate the biochemical chain of reactions termed phototransduction (1). The regeneration of the visual pigments requires that *all-trans*-retinylidene be hydrolyzed and that *all-trans*-retinal be converted back to the 11-cis-configuration through a biochemical pathway referred to as the retinoid cycle (reviewed in ref. 2). First, the chromophore is released from the opsin and reduced in the photoreceptor by retinol dehydrogenases. The product, *all-trans*-retinol, is trapped in the adjacent retinal pigment epithelium (RPE) in the form of insoluble fatty acid esters in subcellular structures known as retinosomes (3).

The isomerization process has so far eluded molecular characterization. The "isomerohydrolase" hypothesis proposes the existence of an enzyme that would use the energy of retinyl ester hydrolysis to carry out the endothermic isomerization reaction (4). This mechanism entails a nucleophilic attack at the C_{11} position of *all-trans*-retinyl palmitate with concomitant elimination of palmitate by alkyl cleavage (Fig. 1*A*). The complex rotates to reposition the C_{11}-C_{12} bond into a new conformation, followed by rehydration of the transition state of the chromophore–protein complex, leading to the production of 11-*cis*-retinol. There is a lack of direct evidence for this mechanism, and its pros and cons have been extensively discussed (5). An alternative mechanism in which *all-trans*-retinyl esters are converted into an unidentified intermediate (*all-trans*-retinol, a subpopulation of activated esters, or an unknown retinoid intermediate) has been proposed by our laboratory (6). This intermediate is then converted to retinyl carbocation, rehydrated in the transition state, and released as 11-*cis*-retinol (7) (Fig. 1*B*). Significant product formation in this endothermic reaction should be seen only in the presence of retinoid-binding proteins (8), and the ratio of the isomers produced appears to be sensitive to the specificity of the retinoid-binding proteins (6, 7). In both mechanisms, the pathway would progress through an alkyl cleavage as observed experimentally (review in ref. 5). Based on the second mechanism, potent transition state analogs could be designed to inhibit the isomerization reaction. Positively charged

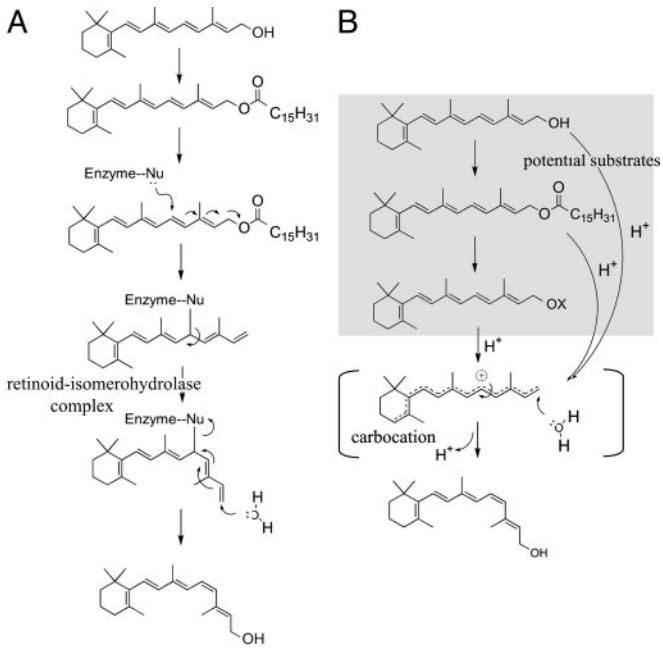

Fig. 1. Two proposed mechanisms of 11-*cis*-retinol formation. (*A*) The first mechanism uses the energy of ester hydrolysis to drive the unfavorable isomerization of *all-trans*-isomer to its thermodynamically less stable 11-*cis*-isomer in a reaction catalyzed by the putative isomerohydrolase (4). (*B*) The second mechanism proposes the formation of 11-*cis*-retinol through a carbocation intermediate where *all-trans*-retinol, *all-trans*-retinyl ester, or another *all-trans*-retinoid derivative becomes protonated, followed by an elimination reaction yielding a retinyl carbocation. Hydration of the carbocation leads to the formation of 11-*cis*-retinol. The reaction is catalyzed by an unknown isomerase and energetically driven by mass action of binding proteins (5).

Abbreviations: CRALBP, cellular retinaldehyde-binding protein; ERG, electroretinogram; LRAT, lecithin:retinol acyltransferase; RA, retinoic acid; RAR, retinoic acid receptor; Ret-NH_2, retinylamine; RPE, retinal pigment epithelium; RXR, retinoid-X receptor.

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retinoids would inhibit the enzyme if the reaction occurred through the carbocation intermediate, whereas the isomerohydrolase mechanism should be much less affected.

Here, we present evidence that the isomerization reaction is potently and reversibly inhibited by a positively charged retinylamine (Ret-NH_2) in *in vitro* assays and in mice. Significantly, Ret-NH_2 severely attenuates rod function while preserving cone-driven electrophysiological responses. These studies provide supportive evidence that the proposed carbocation mechanism is involved in the isomerization process.

Materials and Methods

Animals. All animal experiments used procedures approved by the University of Washington Animal Care Committee. Typically, 6- to 8-week-old mice were used in all experiments.

Materials. Fresh bovine eyes were obtained from a local slaughterhouse (Schenk Packing, Stanwood, WA). Preparation of bovine RPE microsomes was performed according to methods described in ref. 6. All chemicals were purchased from Sigma-Aldrich. 11-*cis*-Retinal was obtained from R. Crouch (Medical University of South Carolina, Charleston, SC).

Retinoid Preparations. All retinoids were purified by normal phase HPLC (Beckman Ultrasphere Si 5μ 4.5 $\times$ 250 mm, 10% ethyl acetate/hexane; detection at 325 nm), and their concentrations in EtOH were determined spectrophotometrically (Fig. 5, which is published as supporting information on the PNAS web site). Absorption coefficients for Ret-NH_2s were assumed to be equal to those of retinol isomers (9, 10).

Chemical Synthesis. Ret-NH_2 was prepared similarly to the method described in ref. 11. N-substituted *all-trans*-Ret-NH_2s were prepared as described above, but instead of NH_3, an excess of the corresponding alkylamine was added to the solution of *all-trans*-retinal in EtOH. *N*-alkyl-Ret-NH_2s were purified on an HPLC column by using the conditions described above. Hydroxylamine derivatives were prepared by the reaction of retinal with the corresponding hydroxylamines in EtOH. *All-trans*-retinal oximes were extracted with hexane, dried, redissolved in EtOH:MeOH (1:1) with an addition of acetic acid (10% vol/vol), and reduced with $NaBH_3CN$. Retinyl amides were prepared by the reaction between *all-trans*-retinylamine and an excess of either acetic anhydride or palmitoyl chloride in anhydrous dichloromethane in the presence of N,N-dimethylaminopyridine at 0°C for 30 min. After the reaction was complete, water was added and the product was extracted with hexane. The hexane layer was washed twice with water, dried with anhydrous magnesium sulfate, filtered, and evaporated. Mass analyses of synthesized retinoids were performed by using a Kratos Analytical Instruments profile HV-3 direct probe mass spectrometer.

Reaction Conditions for Isomerase and Lecithin:Retinol Acyltransferase (LRAT) Reaction. The isomerase reaction was performed essentially as described in ref. 6. The reaction was carried out in 10 mM BTP buffer, pH 7.5/1% BSA, containing 1 mM ATP and 6 μM apo-cellular retinaldehyde-binding protein (CRALBP). To investigate inhibition properties of Ret-NH_2 and its derivatives, RPE microsomes were preincubated for 5 min in 37°C with the indicated compound in 10 mM BTP buffer, pH 7.5/1% BSA/1 mM ATP before the addition of apo-CRALBP and *all-trans*-retinol (to a final concentration of 10 μM unless otherwise indicated). Ret-NH_2 and its derivatives were delivered to the reaction mixture in 1 μl of dimethylformamide (DMF), and the same volume of DMF was added to the control reaction. Each experiment was performed three times in duplicate. The average values were used, and the standard deviations were calculated.

Mouse Retinoid Extraction and Analysis. Retinoid analysis was performed under dim red light as described in refs. 12 and 13. Mice were gavaged with retinoids as described in ref. 13.

Electroretinograms (ERGs). Mice were prepared, and ERG recording was performed as published in ref. 14. Single-flash stimuli had a range of intensities (-3.7–2.8 log cd·s·m^{-2}). Typically, three to four animals were used for the recording of each point in all conditions. Statistical analysis was carried out by using the one-way ANOVA test.

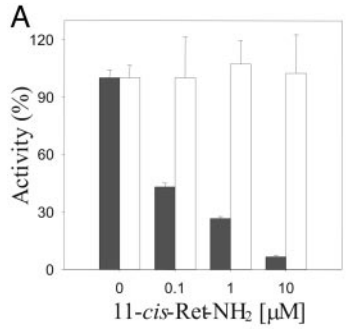
Results

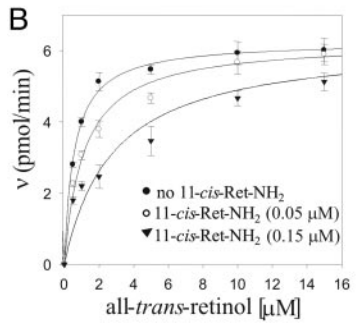
Ret-NH_2 Is a Potent and Specific Inhibitor of the Isomerization Reaction. An *in vitro* assay was used to test whether 11-*cis*-Ret-NH_2 affects isomerization and/or esterification of *all-trans*-retinol. Purified (and irradiated) RPE microsomes were the source of the visual cycle enzymes, and exogenous *all-trans*-retinol was the substrate. In this assay, the amount of both of the *all-trans*-retinyl esters formed in the reaction catalyzed by LRAT as well as 11-*cis*-retinol production by isomerase were measured (6). 11-*cis*-Ret-NH_2 potently inhibited 11-*cis*-retinol production with IC_{50} = 70 nM, whereas the formation of esters was not affected (Fig. 2*A*). Inhibition of isomerization by Ret-NH_2 was not due to competition with 11-*cis*-retinol for binding to CRALBP, as demonstrated by the fact that an increase in the CRALBP concentration from 6 to 30 μM had no effect on the production of 11-*cis*-retinol. In addition, analysis of retinoids bound to CRALBP isolated from the reaction mixture in the presence of the inhibitor did not reveal any significant levels of 11-*cis*-Ret-NH_2 (data not shown). An increased concentration of *all-trans*-retinol lowered the level of inhibition, as illustrated in Fig. 2*B*; however, experiments were performed in a complex system in which numerous enzymatic reactions occur simultaneously with isomerization. Some of these reactions may have a significant influence on the observed kinetics of the reaction. Thus, the type of inhibition could not be determined unequivocally. Nonetheless, these data reflect the potent nature of the inhibition by 11-*cis*-RNH_2.

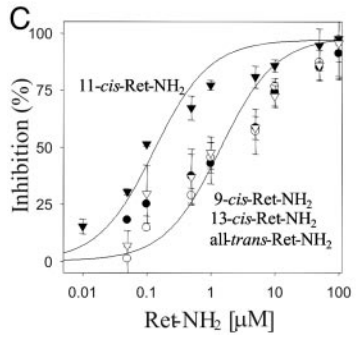
HPLC analysis indicated that the amount of Ret-NH_2 at the start of the assay and at the end was unchanged (within 5% because of N-retinylpalmitamide formation), and no intermediate other than noninhibitory N-retinylpalmitamide (see below) was formed in the time course of the *in vitro* experiments (data not shown). This observation suggests that Ret-NH_2, and not one of its metabolites, is the inhibitor of the isomerization reaction.

Different isomers and derivatives of Ret-NH_2 were synthesized to test the specificity of the inhibition. 11-*cis*-Ret-NH_2 isomer (Fig. 2 *C* and *D*, compound I) was the most potent inhibitor, whereas 9-*cis*-, 13-*cis*-, and *all-trans*-Ret-NH_2 had lower levels of potency with IC_{50} = 640, 730, and 500 nM, respectively (Fig. 2 *C* and *D*, compounds II, III, and IV). An acyclic analog of Ret-NH_2 had lower but comparable potency to 11-*cis*-Ret-NH_2 with 75% inhibition of isomerase at 10 μM concentration (Fig. 2*D*, compound V). *N*-hydroxyretinylamine (reduced oxime; Fig. 2*D*, compound VI) had higher IC_{50}. Interestingly, saturation of the C_{13-14} double bond lowered the potency by $\approx$10-fold for 11-*cis*- and *all-trans*-isomers, suggesting that the presence of the double bond in β-position of an amine is critical for inhibition of the isomerization reaction (Fig. 2*D*, compounds VII and VIII). *N*-alkylated and amide derivatives of Ret-NH_2 failed to effectively inhibit isomerization, suggesting that these analogs do not fit optimally to the binding pocket of the isomerase (Fig. 2*D*, compounds IX–XIII). Ret-NH_2 and its derivatives are not substrates for the isomerase (data not shown). The inhibition was specific to the amino derivatives, because neither *all-trans*-thioretinol nor *all-trans*-13,14-dihydroretinol influenced the isomerization (data not shown).

The specificity of Ret-NH_2 was further tested by biochemical assays. We found that the inhibitor binds to RPE protein(s), but it does not bind to the most abundant protein of the RPE, RPE65.







D

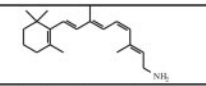
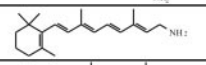
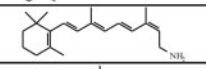
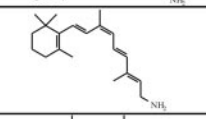
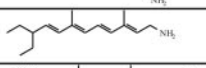
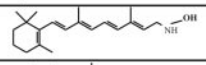
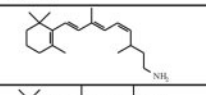
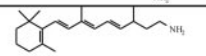
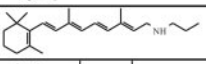
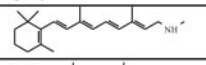
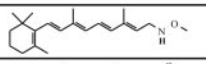
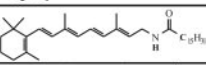
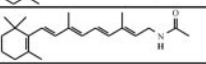
	Retinylamine derivative	Isomerase activity (%) Inhibitor [μM] 1	10	100
I		27 ± 6	13 ± 5	2 ± 3
II		50 ± 10	25 ± 4	7 ± 5
III		52 ± 12	26 ± 7	6 ± 4
IV		52 ± 10	25 ± 6	6 ± 3
V		53 ± 10	18 ± 6	5 ± 3
VI		60 ± 10	23 ± 4	8 ± 1
VII		65 ± 9	32 ± 7	7 ± 2
VIII		80 ± 8	60 ± 5	20 ± 2
IX		80 ± 7	77 ± 5	42 ± 3
X		85 ± 2	80 ± 5	46 ± 4
XI		90 ± 4	71 ± 3	54 ± 6
XII		96 ± 10	89 ± 3	30 ± 9
XIII		99 ± 4	78 ± 8	18 ± 9

Fig. 2. Inhibition of 11-*cis*-retinol isomerase activity by Ret-NH_2 and its derivatives. (*A*) 11-*cis*-Ret-NH_2 inhibits isomerase activity in low-micromolar concentrations *in vitro* without affecting LRAT activity. Filled and open bars correspond to relative amounts of 11-*cis*-retinol and *all-trans*-retinyl esters, respectively, as a function of increased concentration of 11-*cis*-Ret-NH_2. Experiments were performed at a fixed 10 μM *all-trans*-retinol. (*B*) Relation between initial reaction rate (v) and increasing concentrations of *all-trans*-retinol and 11-*cis*-Ret-NH_2. Experimental points were fitted by using classical Michaelis–Menten kinetics. The plotted graph indicates that inhibition of isomerase by Ret-NH_2 is reversible and can be competed out by *all-trans*-retinol. (*C*) Potency of the isomerase inhibition by different Ret-NH_2 isomers. The reaction was carried out with 10 μM *all-trans*-retinol. (*D*) Inhibition of 11-*cis*-retinol production in the isomerase assay by Ret-NH_2 analogs. The reaction was carried out with 10 μM *all-trans*-retinol and described in *Materials and Methods*.

When the RPE microsomes were solubilized with 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine, the isomerase activity decreased only slightly. Then [^{3}H]Ret-NH_2 was added to the RPE extract and loaded on a gel filtration column. Proteins were fractionated and eluted between fractions 18 and 60 (Fig. 3*A*), and the radioactivity was eluted in fractions 36–38. RPE65 was identified by immunoblotting in fractions 23–34 (Fig. 3*B*). The most abundant proteins in this fraction were identified by mass spectrometry and included glucose phosphate isomerase, D-glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, triose phosphate isomerase, enolase, annexin V, calbindin 2, and LRAT. This radioactive elution profile suggests that [^{3}H]Ret-NH_2 formed a complex with a protein(s) other than RPE65. LRAT eluted in most fractions of the chromatogram, suggesting that multiple oligomeric protein–detergent complexes were present in the extract (Fig. 3*B*). In control experiments, retinoids that weakly inhibit the isomerization reaction, such as retinoic acid, *all-trans*-retinol, and Ret-NH_2 acetyl amide (compound XIII), eluted either in the void volume or with low molecular weight compounds. However, a potent inhibitor, an acyclic analog of Ret-NH_2 (compound V), eluted similarly to Ret-NH_2 in fractions 36–38. Moreover, when heat-denaturated proteins were used, [^{3}H]Ret-NH_2 eluted in fractions 48–60, suggesting that specific binding requires nondenatured proteins. In direct assays, Ret-NH_2 did not inhibit retinol dehydrogenases or retinyl acetate hydrolase (5), which are present in the RPE (data not shown).

Ret-NH_2 Affects Recovery of the Chromophore and Visual Functions in Treated Mice. Ret-NH_2 specifically inhibits regeneration of the 11-*cis*-retinal in mice after exposure to intense illumination. In this experiment, we chose *all-trans*-Ret-NH_2 as an inhibitor because it is more stable and available than the 11-*cis*-isomer. Mice were gavaged with *all-trans*-Ret-NH_2 24 h before experiments, exposed to intense light for 20 min, and then allowed to recover in the dark for 5 h. The total amount of different forms of retinoids in all experiments was unchanged. In treated mice, only a residual amount of the 11-*cis*-retinal was present (peaks 2 and 2′, Fig. 4*A Middle*), whereas in untreated mice 11-*cis*-retinal recovered completely (peaks 2 and 2′, Fig. 4*A Top*). Levels of *all-trans*-retinyl esters were elevated in treated animals (Fig. 4*A Middle*, peak 1) as

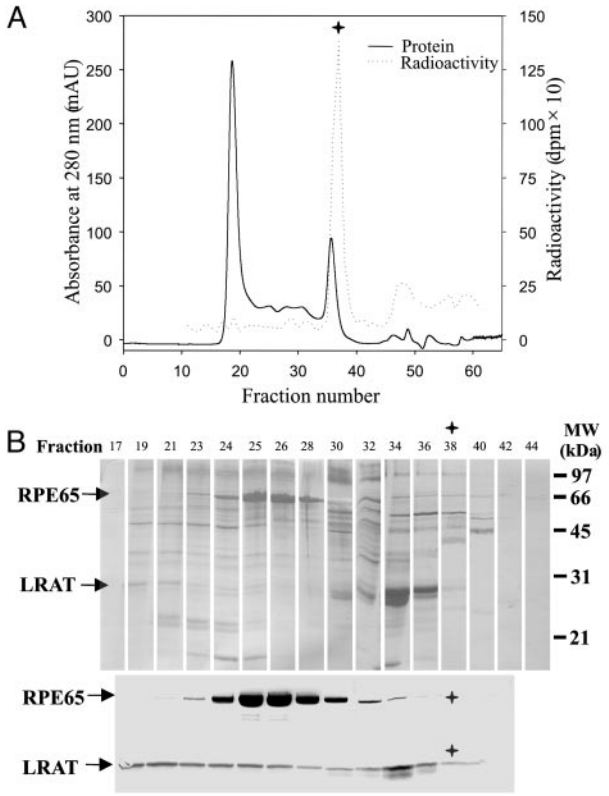


Fig. 3. Gel filtration chromatography of RPE proteins. RPE microsomes were solubilized with 10 mM 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine, incubated with 11,12-di[^{3}H]*all-trans*-Ret-NH$_2$ (1 μM), and loaded on a Superdex 200 column equilibrated with Tris·HCl buffer, pH 7.5, containing 4 mM 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine. Proteins were eluted with a constant flow rate of 0.4 ml/min. The protein and radioactivity levels of 0.4-ml fractions were examined by SDS/PAGE and scintillation counting (*A*), and immunoblotted with anti-RPE65 and anti-LRAT antibodies (*B*). +, the fraction associated with maximum radioactivity.

compared with controls, indicating that LRAT activity is not affected *in vivo*, in accordance with our *in vitro* results. Treatment with *all-trans*-Ret-NH$_2$ did not affect the uptake, transport, or oxidation of 9-*cis*-retinol in treated animals (Fig. 4*A Bottom*). For this experiment, mice were treated with both 9-*cis*-retinol and *all-trans*-Ret-NH$_2$, bleached after 24 h, and allowed to recover for 5 h in the dark. The presence of 9-*cis*-retinal was confirmed in treated animals (see peaks 4 and 4′ in Fig. 4*A Bottom*, corresponding to *syn*- and *anti*-9-*cis*-retinal oximes, respectively). 11-*cis*-Retinal was not reduced and/or esterified after treatment with Ret-NH$_2$. This finding suggests that, as expected, *all-trans*-Ret-NH$_2$ did not compete with the chromophore for the binding pocket of rhodopsin, oxidation/reduction by the RPE, photoreceptor dehydrogenases, or transport to and from the RPE. This finding supports the idea that Ret-NH$_2$ is a specific inhibitor of the isomerization reaction.

The 5 and 24 h dose dependencies of chromophore recovery after Ret-NH$_2$ treatment and a 20 min high-intensity light exposure are shown in Fig. 4*B*. Ret-NH$_2$-treated animals recovered the chromophore much more slowly than untreated animals (IC_{50} = 2 mg/kg for 5 h recovery; Fig. 4*B*, filled circle). The effect of Ret-NH$_2$ on chromophore recovery was reversible, which is demonstrated by the fact that the level of chromophore increased after mice were kept in the dark for 24 h compared with 5 h (IC_{50} = 24 mg/kg for 24 h recovery, Fig. 4*B*, open circle). Mice treated with *all-trans*-Ret-NH$_2$ (50 mg/kg) and not exposed to light had normal levels of retinoids in the eye after 30 h (data not shown). 13-*cis*-RA (retinoic acid, RA) was shown to inhibit the retinoid cycle *in vitro* and in treated animals (15, 16). The potency of Ret-NH$_2$ was tested in parallel with 13-*cis*-RA. In the conditions of our assay, 13-*cis*-RA (50 mg/kg) was ineffective (Fig. 4*B*, red triangles). Ret-NH$_2$ completely blocks recovery of the chromophore after all 11-*cis*-retinal is photoisomerized to *all-trans*-retinal by longer light treatment (Fig. 6, which is published as supporting information on the PNAS web site). Thus, Ret-NH$_2$ is a more potent inhibitor of the isomerization reaction than 13-*cis*-RA.

The conclusions derived from the analysis of retinoids of Ret-NH$_2$-treated mice were supported by ERG analysis of visual functions. ERG responses were significantly affected by a decrease in 11-*cis*-retinal regeneration with *all-trans*-Ret-NH$_2$ administration. Single doses of increasing concentrations of *all-trans*-Ret-NH$_2$ (0.5–100 mg/kg) were delivered to mice via oral gavage. Treated animals did not exhibit symptoms of systemic toxicity such as weight loss or gastrointestinal disorder (data not shown). Next, the impact of *all-trans*-Ret-NH$_2$ administration on the visual physiology of wild-type mice was evaluated *in vivo* with ERGs. Dark-adapted ERGs were performed serially 24 h after *all-trans*-Ret-NH$_2$ gavage (50 mg/kg) and 5 and 24 h after intense bleaching. Treated mice showed normal waveforms and responses in recordings obtained before bleaching. However, after 20 min of constant illumination and 5 h of dark adaptation, a wave and b wave amplitudes from treated mice were significantly attenuated, and they remained so even after 24 h ($P < 0.0001$, one-way ANOVA). In contrast, the dark-adapted state of control mice was fully recovered 5 h after bleaching (Fig. 4 *C* and *D*). The cone function was tested by using photopic ERG conditions. Recovery of cone function was not affected by *all-trans*-Ret-NH$_2$ to the same extent as rod function (Fig. 7, which is published as supporting information on the PNAS web site). This result possibly stems from the fact that cones are able to recover chromophore faster than rods and do not saturate even after an intense bleach, allowing them to operate at low levels of regenerated cone pigments.

13-*cis*-RA can isomerize to *all-trans*-RA and then to 9-*cis*-RA, both of which are potent ligands of the RA receptor (RAR). 9-*cis*-RA also binds to and activates the retinoid-X receptor (RXR). These facts contribute to the toxicity of the RAR and RXR ligands. We investigated whether Ret-NH$_2$ can activate these nuclear receptors by using reporter cells as described in ref. 17. A β-galactosidase activity assay was used to examine whether *all-trans*-RA, 9-*cis*-RA, and Ret-NH$_2$ are agonists for these nuclear receptors. In contrast with *all-trans*-RA and 9-*cis*-RA, *all-trans*-Ret-NH$_2$ was a weak activator of transcription through RA-response elements (Figs. 8 and 9, which are published as supporting information on the PNAS web site).

Next, we investigated the oxidation pathway of *all-trans*-Ret-NH$_2$. Using HEK-293 cells transfected with CYP26A1 cDNA, we found that RA was readily oxidized to 4-oxo-, 4-hydroxy-, and 18-hydroxy-metabolites, whereas there was no evidence of the presence of hydroxy- or oxo-Ret-NH$_2$. This observation suggests that Ret-NH$_2$ is not directly metabolized through the pathway of cytochrome P450 CYP26 enzymes.

In addition to secretion, Ret-NH$_2$ is amidated to N-retinylpalmitamide, as confirmed by HPLC and mass spectrometry analysis of the liver samples of treated mice (data not shown). Unlike *in vitro* experiments, N-retinylpalmitamide is found to be the main metabolite *in vivo*. Analysis of retinoids extracted from livers obtained from mice gavaged with 25 mg/kg Ret-NH$_2$ revealed the presence of 90 μg of amides and 1 μg of Ret-NH$_2$ per 0.5 g of tissue. Observed differences between amide production in the RPE microsomes and *in vivo* can be explained by the disparity in the time course of these experiments and possible differences in the activity of both tissues.

N-retinylpalmitamide (Fig. 2*D*, compound XII) did not inhibit isomerization (Fig. 2*D*), but N-retinylacetamide and N-retinylpalmitamide (Fig. 2*D*, compound XI) were potent inhibitors of

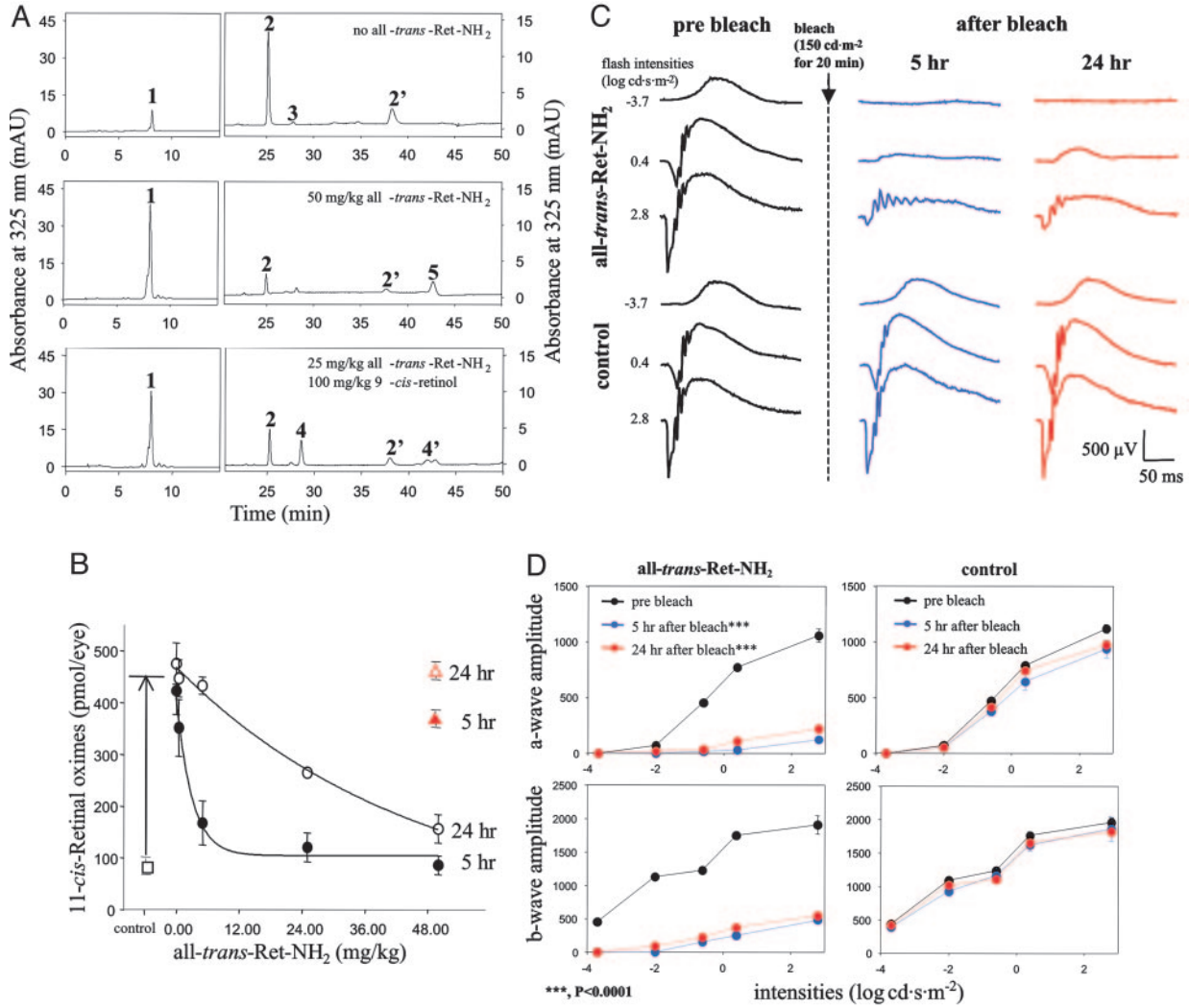


Fig. 4. Ret-NH_2 inhibits regeneration of vision chromophore *in vivo*. (*A*) Chromatographic separation of nonpolar retinoids from WT mouse eyes. Mice were gavaged with *all-trans*-Ret-NH_2 24 h before bleaching for 20 min at 150 cd·m^{-2} W in the Ganzfeld chamber. Regeneration of 11-*cis*-retinal was allowed for 24 h in the dark, and retinoids were extracted from the eye and separated by normal phase HPLC as described in *Materials and Methods*. The peaks were identified based on elution time and absorption spectra and correspond to the following retinoids: 1, *all-trans*-retinyl esters; 2, 2′, *syn*-, and *anti*-11-*cis*-retinal oximes; 3, *syn-all-trans*-retinal oxime; 4, 4′, *syn*-, and *anti*-9-*cis*-retinal oximes; 5, *all-trans*-retinol. (*B*) Changes of 11-*cis*-retinal oxime levels at increasing doses of *all-trans*-Ret-NH_2 during 5 or 24 h of dark adaptation. □, level of 11-*cis*-retinal oximes that was present in mice gavaged with vegetable oil without retinoids just after bleaching. The arrow indicates the increase of visual chromophore during 5 h of dark adaptation. Triangles correspond to the level of 11-*cis*-retinal found in the mice gavaged with 50 mg/kg of 13-*cis*-RA. (*C* and *D*) Single-flash ERG responses of increasing intensity for *all-trans*-Ret-NH_2 treated mice and control mice. Serial responses to increasing flash stimuli were obtained for *all-trans*-Ret-NH_2-treated and control mice for selected intensities (*C*) and plotted as a function of a wave and b wave versus varying light intensities under dark-adapted conditions (*D*) before bleaching and at 5 h and 24 h after bleaching. The dark-adapted mice were bleached with intense constant illumination (150 cd·m^{-2}) for 20 min. The responses from *all-trans*-Ret-NH_2 treated mice were significantly attenuated by single-dose administration (50 mg/kg) compared with control mice ($P < 0.0001$, one-way ANOVA). SE bars are shown.

vision chromophore regeneration in mice (data not shown). This observation and lack of inhibition directly by the amide can be explained by hydrolysis of the N-retinylamides back to Ret-NH_2.

Discussion

A Tool to Study the Mechanism of *all-trans*-Retinol:11-cis-Retinol Isomerization. We have uncovered specific and potent inhibitors of the isomerization reaction of the retinoid cycle. The enzyme that carries out *all-trans*-retinol:11-*cis*-retinol isomerization is not known, but a few different theories have emerged with regard to the mechanism of isomerization. In one model, *all-trans*-retinyl esters are the direct substrate of the isomerohydrolase (4), a hypothetical enzyme that couples the energy of ester hydrolysis with the endothermic isomerization of the $C_{11–12}$ double bond (Fig. 1*A*). A number of observations on the isomerization reaction have brought the isomerohydrolase hypothesis into sharper question (5). To reconcile these discrepancies, we have proposed a second mechanism, which offers a different view regarding the isomerization step. In our proposed mechanism (Fig. 1*B*), an as-yet-unidentified intermediate undergoes protonation at the oxygen atom, which ultimately leads to an elimination of the carboxylic acid, leaving *all-trans*-retinol as a retinyl carbocation. The positive charge is delocalized throughout the conjugated double bonds (Fig. 1*B*). Results presented here suggest that Ret-NH_3^+ might mimic the transition state analog proposed in the carbocation mechanism. Because 11-*cis*-Ret-NH_2 is a more potent inhibitor than *all-trans*-Ret-NH_2, it may be that the retinyl carbocation-enzyme bound structure resembles the 11-*cis*-retinal configuration.

Ret-NH_2 is the most effective inhibitor tested, and modifications of the amino group decrease its potency. An exception is N-

retinylhydroxylamine, which could form a hydrogen bond network created by the substitution of an -NHOH group for the $-NH_3^+$ group and could be protonated in the binding site of the enzyme. Most of the tested retinoids are protonated at neutral pH, and this feature appears to be a prerequisite for potent inhibition. Bulky Ret-NH_2 derivatives, such as *N*-alkyl-Ret-NH_2 or N-retinylpalmitamide, are ineffective inhibitors and most likely do not fit well into the binding pocket of the isomerase. Based on this observation, one may speculate that the real substrate is not a fatty acid retinyl ester, but presumably a more polar compound such as a retinol or a low molecular weight retinyl derivative.

Several observations from our *in vitro* and *in vivo* experiments indicate that Ret-NH_2 is a specific inhibitor of the isomerization process. For example, *in vitro* assays demonstrate that this compound does not inhibit LRAT, retinol dehydrogenases, or retinyl ester hydrolases. Moreover, Ret-NH_2 does not bind to CRALBP or RPE65. *In vivo* results also support the idea that no other step in the retinoid cycle is affected by Ret-NH_2 except the isomerization reaction.

Comparison of Ret-NH_2 to 13-*cis*-RA. The chemistry and biological activity of the previously identified 13-*cis*-RA (18) and Ret-NH_2 inhibitors of the retinoid cycle stand in sharp contrast with each other. The two possible metabolic fates of 13-*cis*-RA are isomerization to *all-trans*-isomer or oxidation followed by glucuronidation and secretion (19). 13-*cis*-RA will occur in equilibrium with *all-trans*-RA, which can activate the RA-dependent transcriptional pathway. RA was shown to activate transcription of target genes by binding to nuclear RARs and RXRs (20). Notably, 13-*cis*-RA is highly toxic during pregnancy (21). There are α, β, and γ isotypes of RAR containing the ligand-binding domains that bind both 9-*cis*-RA and *all-trans*-RA. The ligand-binding domain of the RXR α, β, and γ isotypes binds only 9-*cis*-RA. RAR mediates activation of genes that contain *cis*-acting response elements (RARE) by forming RAR/RXR heterodimers. RARE elements consist of direct repeats of hexameric motifs PuG(G/T)TCA separated by 1–5 bases and have been found in the promoter region of many genes (22). RXR homodimers can be activated by 9-*cis*-RA (23) and other hydrophobic substances. RXR homodimers are specific for DR1 elements of hexameric motifs separated by a single base pair (24). The activation of genes mediated by RAR or RXR can be studied by using reporter genes containing *lacZ* under the control of a minimal promoter and the appropriate DR element located immediately upstream of the reporter gene. In our assay, as expected, 9-*cis*- and *all-trans*-RA activated transcription of DR1 or DR5 RARE elements, whereas Ret-NH_2 was >1,000-fold less active in this assay. In addition, retinylamines can be stored in the form of amide and do not undergo oxidative hydroxylation by Cyp26s (25). The amide storage form is reversible with free amine, explaining the long-lasting inhibition of isomerization by Ret-NH_2 in mice.

Value of a Specific Inhibitor of Isomerization for Understanding of the Retinoid Cycle and Potential Application in Treatment of Retinal Diseases. Identification of a highly potent group of inhibitors will expand studies of the retinoid cycle *in vivo* and *in vitro*. In particular, it is now possible to regenerate rhodopsin with labeled 9- and 11-*cis*-retinals to track the retinoid cycle in rodent eyes. This approach, together with two-photon microscopy (3), will allow us to study the dynamic flux of retinoids through the retinoid cycle in wild-type and genetically engineered mice. These inhibitors also appear to be useful ligands for affinity chromatography during efforts to isolate the isomerization complex.

The use of potent inhibitors of isomerization *in vivo* after light exposure may also prevent or slow down the recovery of visual pigment chromophore production. In Stargardt's disease (26), associated with mutations in the photoreceptor-specific ATP-binding cassette transporter, the accumulation of *all-trans*-retinal is thought to be responsible for the formation of a lipofuscin pigment, 2-[2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E,7E-octatetraenyl]-1-(2-hydroxyethyl)-4-[4-methyl-6(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E,7E-hexatrienyl]-pyridinium) (A2E), which is toxic toward retinal cells and causes retinal degeneration and consequent loss of vision (27, 28). It was proposed that treating patients with an inhibitor of the retinoid cycle, 13-*cis*-RA (Accutane, Roche), might prevent or slow down the formation of A2E and might also have protective properties for normal vision (15). It would be interesting to test whether Ret-NH_2 decreases A2E formation, because both 13-*cis*-retinoic acid and Ret-NH_2 should act by the same mechanism to delay the regeneration of visual pigments. More studies are needed to establish the toxicity and metabolism of Ret-NH_2, but based on our data, it is clear that Ret-NH_2 does not activate transcription of genes at the same level as retinoic acid and its derivatives. Thus, it is reasonable to speculate that the higher potency per dose, the much lower transcriptional activation profile, and the preservation of cone vision together make Ret-NH_2 a highly improved treatment candidate alternative to 13-*cis*-RA.

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